Increased Expression of Cyclooxygenase-2 in Rat Lung Tumors Induced by the Tobacco-specific Nitrosamine 4-(Methylnitrosamino)-4-(3-pyridyl)-1-butanone: The Impact of a High-Fat Diet

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Abstract
Aberrant or excessive expression of cyclooxygenase (COX)-2 has been implicated in the pathogenesis of many disease processes, including carcinogenesis. COX-2 expression was immunohistochemically examined in archival samples (D. Hoffmann et al., Cancer Res., 53: 2758–2761, 1993) of lung neoplasms (adenomas, adenocarcinomas, and adenosquamous carcinomas) induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in male F344 rats that had been fed either a semipurified AIN-76A diet with high-fat (HF; 23.5% corn oil) or low-fat (LF; 5% corn oil) content. The intensity and extent of COX-2 positivity was graded from 0 (undetectable or negligible expression) to grades 1 (<30% expression), 2 (30–60% expression), 3 (60–90% expression), and 4 (>90% expression). The scoring criteria were similar to those used with specimens from human lung cancers (T. Hida et al., Cancer Res., 58: 3761–3764, 1998). In group 1 (NNK plus HF diet), adenomas, adenocarcinomas, and adenosquamous carcinomas were of mean grades 2, 3, and 4, respectively; in group 2 (NNK plus LF diet), the corresponding mean grades were 1, 1, and 3. Although control rats, given HF (group 3) or LF (group 4) diets but no NNK, developed spontaneous lung tumors, the expression of COX-2 was either negligible (one adenoma of grade 0 in group 3) or of a very low grade (one adenocarcinoma of grade 1 in group 4). In addition, the latency of the tumors in the peripheral lung in assays with NNK is significantly shorter in rats maintained on the HF diet than in those on LF diet. COX-2 expression was not evident in normal lung tissues. We report here for the first time that NNK induces increasingly higher levels of COX-2 expression with progressive stages of lung tumorigenesis when rats are fed the HF diet. The increase in COX-2 expression may be associated with the development of lung tumors induced by NNK. This well-defined animal model is valuable for studying modulation of COX-2 expression in lung carcinogenesis by various factors, including dietary components.

Introduction
Smoking of cigarettes, cigars, and pipes is causally related to the risk for cancer of the lung (1). In recent decades, the rate of adenocarcinoma has been increasing relative to that of the formerly common tumor type, the squamous cell carcinoma (2–4). This may reflect changes in cigarette formulation toward lower nicotine yields, which have led smokers to modify their smoking pattern, i.e., taking larger puff volumes while inhaling the smoke more deeply, which enables these carcinogens to reach the peripheral parts of the lung where the adenocarcinoma primarily develops (5). Moreover, changes in cigarette make-up have also led to relatively higher smoke yields of the type of nicotine-derived carcinogenic N-nitrosamines that induce adenocarcinoma in laboratory animals (6). Tobacco-specific nitrosamines represent a class of potent animal lung carcinogens (7, 8). NNK (Fig. 1) is a representative agent of this class. An etiological role of NNK in the causation of human lung cancer is supported by assays showing that human lung tissues can activate NNK to DNA-damaging agents (9) and that the lung is a target organ for NNK-induced carcinogenesis in rodents (reviewed in Ref. 10). NNK induces adenocarcinoma of the lung in mice, rats, and Syrian golden hamsters, independent of the route of administration (reviewed in Ref. 11).

The bioactivation of NNK in rats, mice, Patas monkeys, and several in vitro systems is catalyzed by multiple forms of cytochrome P450 isozymes (10). However, Smith et al. (12) suggested that P450 enzymes are only partially responsible for the activation of NNK in human lung microsomes and that COX and LOX may play important roles in the oxidation of NNK in this organ. This suggestion appears to be further supported by observations made by Rioux and Castonguay (13), who showed that inhibition of COX and LOX expression diminished NNK-induced lung tumorigenesis. COX also catalyzes conversion of certain polynuclear aromatic hydrocarbons and/or metabolites to the ultimate carcinogenic bay region diol epoxide (14, 15). Thus, it appears that COX and LOX enzyme systems are important in metabolic activation of carcinogens in extrahapatic tissues where P450 is not prevalent.

Two isoforms of COX, COX-1 and COX-2, have been identified (reviewed in Refs. 16 and 17). COX-1 is constitutively expressed in most tissues and is thought to be involved in maintaining cellular homeostasis. In contrast, COX-2 is frequently undetectable in nonneoplastic tissues, but it is inducible by phorbol esters, cytokines, growth factor, reactive oxygen species, and chemical carcinogens. For example, benzo[a]pyrene was found to up-regulate COX-2 gene expression in normal and transformed oral epithelial cells in vitro (18). COX-2 but not COX-1 expression is markedly elevated in most colonic tumors in azoxymethane-treated rats and in intestinal neoplasia in Min mice (19, 20). In humans, overexpression of COX-2 has also been associated with colorectal cancer and lung cancers, specifically, adenocarcinomas (21–23).

Epidemiological studies indicate that the fat content of the diet plays an important role in the risk for cancer of the lung (24, 25). Experimentally, the role of dietary fat and its relationship to carcinogenesis has been explored in various settings (26–28). For example, the role of dietary fat in the development of NNK-induced adenoma, adenocarcinoma, and adenosquamous carcinoma in rat lung has been examined (28); the results underscore the likelihood of a dietary role of fat in human cancer that is suggested by epidemiological observations. Using archival rat lung neoplasms, we report here that NNK and HF diet increased COX-2 expression in progressively anaplastic pulmonary neoplasms. Also, COX-2 levels appear to rise early when
NNK is given with a HF diet, which shortens the latency of lung tumor development in rats.

Materials and Methods

Source of Archival Lung Tumor. Our bioassay has been described previously (28). It consisted of the following groups: group 1, 23.5% corn oil (HF) diet, 2 ppm NNK, 60 rats (NNK-HF); group 2, 5% corn oil (LF) diet, 2 ppm NNK, 60 rats (NNK-LF); group 3, 23.5% corn oil diet, tap water, 20 rats (HF); group 4, 5% corn oil diet, tap water, 20 rats (LF). Groups 3 and 4 were negative controls. The NNK-containing drinking water was offered throughout the lives of the rats, beginning when they were 8 weeks of age. The animals were observed until they were moribund or until the scheduled termination of the experiment (when 90% of the rats in the longest surviving group had died), when the rats were killed by CO2 asphyxiation. Complete autopsies were performed, and H&E-stained microscopic slides were prepared for histopathological evaluation of all gross lesions in the lung.

Immunohistochemistry. The immunohistochemistry method used was modified from a previously published study (29). Four-μm-thick sections of formalin-fixed and paraffin-embedded tissue samples from gross lesions of the lung were microwaved in citrate buffer (pH 6), endogenous peroxidase was blocked with 3% hydrogen peroxide, and tissues were incubated with Universal Protein Blocker (Shandon Lipshaw, Pittsburgh, PA). Subsequently, tissues were incubated with primary antibody of prostaglandin H synthase 2 (COX-2), purchased from Caymen Chemical (Ann Arbor, MI). At a dilution of 1:50, the incubation was for 1 h at room temperature, followed by staining with biotinylated goat antirabbit Vector Elite Kit (Vector Laboratories, Burlingame, CA). Tissues were then incubated with strepavidin horseradish peroxidase biotinylated goat antirabbit Vector Elite Kit (Vector Laboratories, Burlingame, CA). Tissues were then incubated with strepavidin horseradish peroxidase enzyme (Lab Vision Corp., Freemont, CA) at room temperature for 20 min, followed by 3-aminio-9-ethylcarbazole (AEC) chromogen for 7 min at room temperature.

Evaluation of COX-2 Immunostaining. The intensity and extent of COX-2 positivity was graded in a blinded fashion with coding. Lung tissue samples showing undetectable or negligible expression of COX-2 were scored as grade 0. The intensity and degree of positive reaction were further scored as follows: grade 1, <30% positivity; grade 2, 30–60% positivity; grade 3, 60–90% positivity; and grade 4, >90% positivity. The scoring criteria were similar to those used with human specimens (21).

Results and Discussion

COX-2 expression was examined in archival samples of lung neoplasms (adenomas, adenocarcinomas, and adenosquamous carcinomas) induced by NNK in male F344 rats fed either HF or LF diets (28). The data presented in Table 1 summarize the total incidence of each tumor type and the mean grade (intensity/severity) of COX-2 expression, as assessed by immunohistochemistry for each tumor type. Furthermore, the latency (time to lethal tumor), expressed in weeks (mean ± SD), is also presented in Table 1. The results reported here indicate for the first time that NNK causes increasingly higher levels of COX-2 expression with progressive stages of lung carcinogenesis in rats fed a HF diet and that COX-2 levels rise earlier, shortening tumor latency in rats maintained on NNK and HF diet compared to rats exposed to NNK and a LF regimen.

Morphologically, NNK-induced lung neoplasms were broncholaveolar adenomas, bronchioalveolar carcinomas, and/or adenosquamous carcinomas. All of these neoplasms showed COX-2-positive expression in the interstitium, in the case of adenomas (Fig. 2, A and B); within the cells of papillary growth in carcinomas (Fig. 2, C–F); or even within squamous cell clusters in adenosquamous carcinomas (Fig. 2, G and H). The results presented in Table 1 reflect that, in group 1 (NNK plus HF diet), adenomas, adenocarcinomas, and adenosquamous carcinomas were of mean grades 2, 3, and 4, respectively. Group 2 (NNK plus LF) neoplasms were of mean grades 1, 1, and 3, respectively. Thus, in group 1, most adenomas were of grade 2 and most adenocarcinomas were of grade 3, whereas the corresponding neoplasms in group 2 were of grades 1 and 1, respectively.

In the spontaneous lung neoplasms observed in control rats [groups 3 (HF) and 4 (LF)], the expression of COX-2 was either negligible (one adenoma of grade 0 in group 3) or of very low grade (one adenocarcinoma of grade 1 in group 4). Notably, nonneoplastic lung tissues had no measurable COX-2 expression. Conversely, in all NNK-induced lung neoplasms, there was an elevation in COX-2 expression that may be associated with the development of lung tumors induced by NNK in rats. Thus, these results, together with those reported previously on the increased expression of COX-2 in human lung cancer, suggest that the well-defined NNK lung tumor model assay will become valuable for studying pulmonary neoplasia using COX-2 as a marker. However, the dietary regimen (fat content of diet), both in model assays and in humans, appears to be most important.

Of particular significance is the latency of these neoplasms, i.e., the time to lethal tumor development for each specific type. The latency (in weeks) for lung adenomas (mean ± SD) was 78.4 ± 10.9 in group 1, whereas the corresponding time in group 2 was 89.8 ± 5.6 (P < 0.05). In group 1, the adenomas were evident at ~11 weeks before the adenomas in group 2. For adenocarcinomas, the latency in group 1 (83.5 ± 8.7) was significantly different from that in group 2 (91.8 ± 7.8) at P < 0.01. The one adenosquamous carcinoma in group 1 was detected at 77 weeks, whereas the same type adenosquamous carcinoma in group 2 was detected at 92 weeks; again, 15 weeks after

Table 1 Mean COX-2 expression over time in three types of lung neoplasia induced by NNK in male F344 rats

<table>
<thead>
<tr>
<th>Treatment (no. of rats)</th>
<th>Experimental group no.</th>
<th>No. of lung tumors (mean COX-2 expression)</th>
<th>Tumor latency, weeks (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNK-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HF (60)</td>
<td>1</td>
<td>Ad: 19 (2) Ac: 15 (3) As: 1 (4)</td>
<td>Ad: 78.4 ± 10.9 Ac: 83.5 ± 8.7 As: 77</td>
</tr>
<tr>
<td>LF (60)</td>
<td>2</td>
<td>Ad: 4 (1) Ac: 19 (1) As: 1 (3)</td>
<td>Ad: 89.8 ± 5.6 Ac: 91.8 ± 7.8 As: 92</td>
</tr>
<tr>
<td>Vehicle-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HF (20)</td>
<td>3</td>
<td>Ad: 1 (0) Ac: NA As: NA</td>
<td>83 Ad: NA Ac: NA As: NA</td>
</tr>
<tr>
<td>LF (20)</td>
<td>4</td>
<td>Ad: NA Ac: 1 (1) As: NA</td>
<td>Ad: NA Ac: 96 As: NA</td>
</tr>
</tbody>
</table>

* The types of lung tumors observed in this study were adenomas (Ad), adenocarcinomas (Ac), and adenosquamous carcinomas (As).

** Total number of specific lung tumors (mean grade of COX-2 in this type of lung neoplasm, scored as described in “Materials and Methods”).

* Group 1 is significantly different from 2 upon comparing the same type of tumors at P < 0.05; Student’s t test.

** Group 1 is significantly different from 2 upon comparing the same type of tumors at P < 0.01; Student’s t test.

NA, not applicable.
that in group 1. In the control groups, the adenoma was evident at 83 weeks [group 3 (NNK plus HF)] and the adenocarcinoma [group 4 (NNK plus LF)] at 96 weeks.

It is evident that inhibition of COXs retards tumorigenesis in animals, as was first demonstrated with nonsteroidal anti-inflammatory drugs, such as acetylsalicylic acid (aspirin) and sulindac, which inhibit both COX activity and NNK-induced lung tumorigenesis in A/J mice (30). It is worth noting that, in addition to the observation in A/J mice, an association between aspirin consumption and reduced lung cancer incidence in men was also reported (31). Thus, it is essential to understand the mechanisms by which NNK or other carcinogens up-regulate COX-2 and how dietary fat modulates these effects, so that specific modes of intervention can be developed while critical signals that mediate the induction of COX-2 expression are monitored. Such investigations will be the focus of our future studies.

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References

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